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Redox regulation of peroxiredoxin and proteinases by ascorbate and thiols during pea root nodule senescence

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Abstract **Redox factors contributing to nodule senescence were studied in pea. The abundance of the nodule cytosolic peroxiredoxin but not the mitochondrial peroxiredoxin protein was modulated by ascorbate. In contrast to redox-active antioxidants such as ascorbate and cytosolic peroxiredoxin that decreased during nodule development, maximal extractable nodule proteinase activity increased progressively as the nodules aged. Cathepsin-like activities were constant throughout development but serine and cysteine proteinase activities increased during senescence. Senescence-induced cysteine proteinase activity was inhibited by cysteine, dithiotreitol, or E-64. Senescence-dependent decreases in redox-active factors, particularly ascorbate and peroxiredoxin favour decreased redox-mediated inactivation of cysteine proteinases.**

Keywords: Senescence; Proteinase; Ascorbate; Peroxiredoxin; Redox signalling

1. Introduction

Nodule senescence, like leaf senescence [1] is a highly organised process, orchestrated in an age-dependent manner [2]. However, nodule longevity is also determined by environmental factors. High nitrate availability or stressful environmental conditions such as high temperatures, acid soil conditions, root pathogen infection or drought induce premature senescence [3–6]. Characteristic features of nodule senescence are loss of leghemoglobin and N_2 fixation and increased lytic activities [7–9]. The nodule cytosolic proteins, especially leghemoglobin, are considered to be early targets of senescence-induced proteinases [10–12].

Cysteine proteinases are involved in nearly every stage of plant development from the degradation of seed storage proteins to fruit ripening as well as in legume nodule development. They are important in stress responses as well as in programmed cell death. Some cysteine proteinases have specific characteristics such as a C-terminal KDEL motif, which is an endoplasmic reticulum retention signal for soluble proteins. In pea, transcripts encoding a cysteine proteinase denoted as *PsCyp15a*, accumulated first in wilting leaves [13,14] and then in nodules [15,16]. A second cysteine proteinase denoted as *PsCyp1* was reported to be more highly expressed in nodules than in shoots [15]. A nodule-specific and senescence-related cysteine proteinase belonging to the legumain family has been identified in soybean [17]. Three groups of cysteine proteinases were expressed in the senescing zone of white clover nodules [18].

Micro- and macroarray studies on mature *Medicago truncatula* nodules have indicated a complex picture of up- and down-regulated genes from the same proteinase families. In particular, the proteasome subunit β type and the ubiquitin-conjugating enzyme E2 were up-regulated while a cysteine proteinase (26S protease regulatory subunit 4) was down-regulated [19]. An increasing body of evidence suggests that the activity and function of the ubiquitin-dependent 26S proteasome protein degradation pathway is controlled by cellular redox status of the cell. A high cellular redox status, as defined for example by high GSH/GSSG ratios, impedes the binding of target proteins [20–23]. Nodule senescence is characterised by decreased levels of ascorbate and glutathione [2,6,24–27], a condition that would tend to favour the activation of the 26S proteasome protein and enhance protein degradation.

Peroxiredoxins constitute small gene families in all organisms including cyanobacteria and plants [28,29]. They have recently been identified as important components of the plant antioxidant defence network. They detoxify alkyl hydro peroxides, hydrogen peroxide and peroxinitrite. In addition to their role in antioxidant defence they appear to control or modulate redox dependent signalling. In *Arabidopsis thaliana*, four peroxiredoxins are targeted to the chloroplast (2-Cys Prx A and B, Prx Q and Prx II E), one to the mitochondrion (Prx II F), one to the nucleus (1-Cys Prx) and four remain in the cytosol (Prx II A–D). Plants lacking mitochondrial peroxiredoxin

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Abbreviations: Asc, ascorbate; DTT, dithiothreitol; Prx, peroxiredoxin; PMSF, phenylmethylsulphonylfluoride; E-64, L-*trans*-epoxysuccinyl-laucylamido(-guanidino) butane

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(Prx) II F have altered gene expression for example of antioxidant defence genes, and root growth is strongly inhibited under stress [30]. The role of the cytosolic peroxiredoxins remains uncharacterised, but expression analysis suggests that these isoforms function during optimal conditions as well as under stress [31,32]. To date, there have been no reports of nodule peroxiredoxins and their role in nodule development and function remains to be elucidated.

We have previously reported that the activities of many of the pea nodule antioxidant enzymes as well as the GSH/GSSG ratio are not decreased greatly at the time of nodule senescence [27]. Hence, here we have concentrated in the present experiments, on key nodule antioxidants particularly ascorbate, which show marked changes consistent with senescence. Since peroxiredoxins are essential contributors to the redox homeostasis of the plant cell, the occurrence of peroxiredoxins and their abundance was studied during nodule development. The following experiments were therefore undertaken to determine the extent to which redox factors influence pea root nodule senescence. We analysed nodule proteinase activities and antioxidants, particularly ascorbate and cytosolic peroxiredoxin. We show that pea redox-sensitive proteinases are inhibited by reductant and that the abundance of cytosolic peroxiredoxin is modulated by ascorbate. These results point towards the importance of redox factors in the control of nodule senescence.

2. Materials and methods

2.1. Plant material

Pea seeds (*Pisum sativum* cv. Phönix) were inoculated with a commercial *Rhizobium* strain (HiStick pea inoculants, Becker Underwood Ltd., USA). Plants were grown on vermiculite in controlled environment chambers with a 25 °C/19 °C, 70%/85% humidity day/night regime, under a 14-h photoperiod (600 μmol m^{-2} s^{-1}). Plants were supplied daily with nitrogen-free nutrient solution [33].

2.2. Chlorophyll content, total soluble protein and nitrogenase activity measurements

Chlorophyll was determined in leaf extracts prepared in 80% acetone according to [34]. For estimations of protein and nitrogenase activity tissues were homogenised in 50 mM Tris buffer (pH 8.0) and centrifuged for 15 min at 17000 × *g*. The total protein content of the supernatant was estimated with BioRad protein microassay (Bio-Rad, Richmond, CA, USA). Nitrogenase activity was assayed by the acetylene reduction method [35] using excised nodules as described by Groten et al. [27].

2.3. Nodule proteinase activities

Nodule proteinase activity was assayed by a standard fluorimetric microassay method [36–38] using specific substrates for cathepsin L (N-CB2-phe-arg-MCA, Sigma–Aldrich, UK), cathepsin B (Nα-CB2-arg-arg-MCA, Sigma–Aldrich, UK) and cathepsin H (L-arg-MCA, Sigma–Aldrich, UK). Azocaseinase activities were measured as described previously [9,39,40]. In-gel detection of proteinase activity was performed according to the method of Michaud et al. [41]. Proteins (5 μg) were separated by mildly denaturing gelatine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). In order to discriminate between cysteine and serine proteinases, protein extracts were pre-incubated for 15 min at 37 °C with 100 μM L-*trans*-epoxysuccinyl-laucylamido(-guanidino) butane (E-64, Sigma–Aldrich) and 1 mM phenylmethylsulphonylfluoride (PMSF, Sigma–Aldrich), class-specific inhibitors of cysteine and serine proteinases respectively. For activation of cysteine proteinases, cysteine (5 mM) and dithiothreitol (DTT, 1 mM), respectively, were added together with the inhibitors.

2.4. Immunoblot analysis of nodule peroxiredoxins

Extracted proteins (50 μg) were denatured with reducing SDS sample buffer, boiled for 10 min and separated by SDS–PAGE. SDS–PAGE and immunoblotting was performed as described previously [32]. Antisera directed against the recombinant *Arabidopsis* type II peroxiredoxins C and F were each used in a 1:10000 dilution.

2.5. Total ascorbate content and ascorbate feeding experiments

Three and 9-week-old nodules were vacuum-infiltrated (30 mbar) for 8 h at room temperature with 10 mM sodium phosphate buffer (pH 7.8) in the presence or absence of 20 mM ascorbate. Samples were then incubated in 10 mM sodium phosphate buffer (pH 7.8) and removed after 8 and 24 h for analysis of peroxiredoxin protein abundance. Total ascorbate and reduced ascorbate contents were measured in neutralised root and nodule extracts at the indicated time-points as described previously [42] by measuring the decrease in absorbance at 265 nm before and after the addition of ascorbate oxidase. For the quantification of total ascorbate, dehydroascorbate was reduced back to ascorbate using DTT. Samples were incubated with 1 mM DTT in the dark for 30 min prior to measurement.

3. Results

3.1. Senescence characteristics

Pea leaf chlorophyll contents (Fig. 1A) declined sharply 9 weeks after sowing. The plants started to flower at 6 weeks after sowing while both leaf and nodule total soluble protein content (Fig. 1B and C) decreased after 9 weeks. Nitrogenase activity declined gradually from 3 weeks after sowing but still maintained high levels in nodules up to an age of 9 weeks (Fig. 1D).

3.2. Proteinase activities increase during nodule senescence

Total proteinase activities, determined either by spectrophotometric assays (Fig. 2A) or by in-gel proteinase activity assays using gelatine as a substrate (Fig. 2B), increased during nodule senescence. Proteinase activities increased in nodules 5–7 weeks after sowing but highest activities were observed in senescent nodules. The in-gel-proteinase assay revealed two major activity bands at approximately 45 kDa and slightly more than 160 kDa. The assay was performed at pH 8 and pH 5. The activity pattern was fairly similar under both conditions (data not shown).

Class-specific proteinase inhibitors were used to discriminate between the types of proteinases induced during nodule senescence. PMSF, an inhibitor of serine proteinases, inhibited the activity of the high molecular weight isoform (Fig. 3A) but not that of the 45 kDa isoform. The activity of the 45 kDa isoform declined following the addition of E-64, an inhibitor of cysteine proteinases (excluding legumain-type proteinases). It is interesting to note that there is also a cysteine proteinase activity that appears early in nodule development with activity at pH 8 (Fig. 3A), suggesting that this is a cytoplasmic enzyme form.

The assays with E-64 routinely contained 1 mM DTT or 5 mM cysteine because cysteine proteinases are generally considered to be activated under reducing conditions. We therefore measured nodule proteinase activities in the presence or absence of DTT and with or without E-64. Surprisingly, DTT strongly decreased proteinase activity in 9-week-old nodules (Fig. 3B). Moreover, nodule cysteine proteinase activities were completely inhibited by the simultaneous application of DTT and E-64 (Fig. 3B).

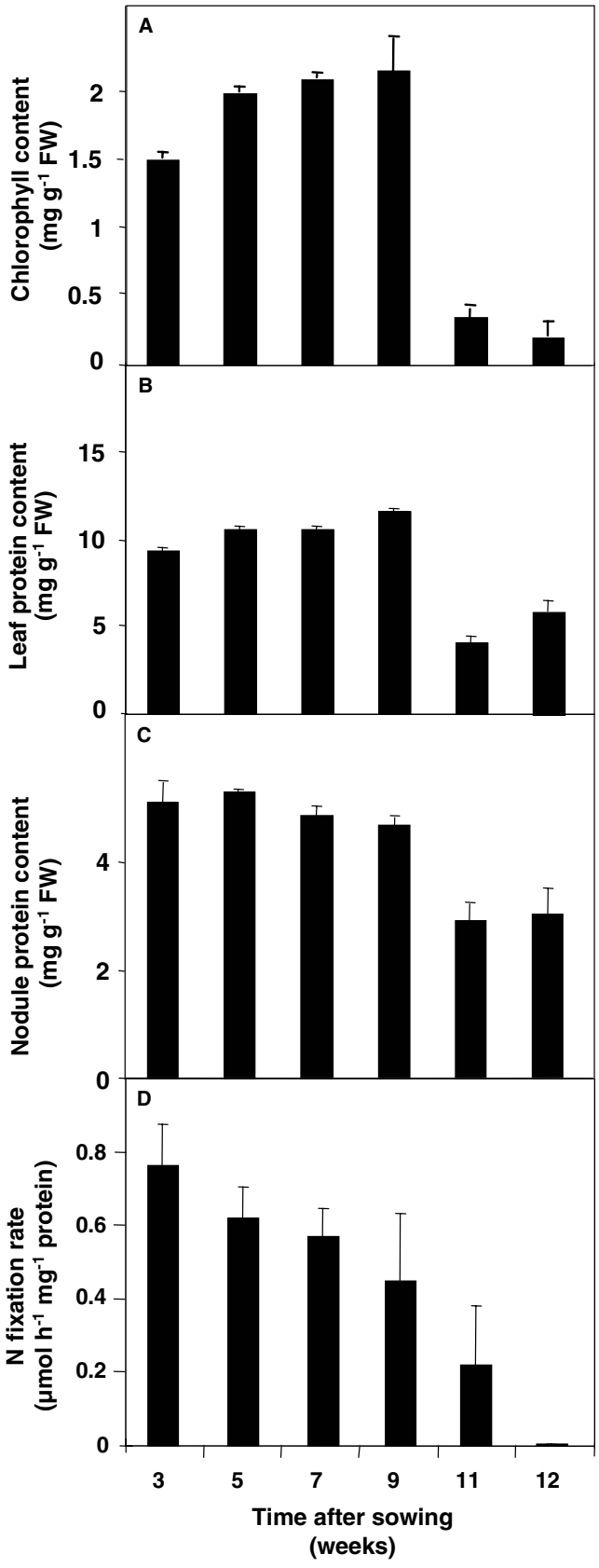

Fig. 1. Changes in leaf chlorophyll (A), leaf protein (B), nodule protein (C) and nodule N fixation rates (D) during plant development. Each column represents the mean of three plants ± SE.

3.3. Nodule cathepsin-like activities

The amino acid sequence of legumain, together with the location of its active site, show that this enzyme belongs to a distinct family of cysteine proteinases unrelated to papain, but rather related to the caspase family [38]. Cathepsin B, H and L-like activities were detected in nodules using specific fluorescent substrates. While cathepsin L- and H-like activities transiently increased during development, cathepsin B-like activities remained fairly constant (Fig. 4A) Even though cathepsin-like activities showed very high activities particularly at more alkaline pH values (Fig. 4B) no relationship was observed between root nodule senescence and cathepsin-like activities at pH 8.0 (Fig. 4A) or at any other pH value measured (data not shown).

3.4. Root and nodule ascorbate and peroxiredoxin content

Total ascorbate contents were much higher in roots (Fig. 5A) than in nodules (Fig. 5B), but declined in both tissues during development. The ratio of ascorbate to dehydroascorbate was low (only 40% reduced at maximum) in roots (Fig. 5A) and also in the nodules (Fig. 5B). Roots are often found to have lower ascorbate to dehydroascorbate ratios than leaves, presumably because a high proportion of the root dehydroascorbate is present in the apoplast where it is not accessible for re-reduction. The increase in the ratio of ascorbate to dehydroascorbate in 7-week-old nodules is intriguing and possible reflects the importance of a high redox state in maintaining a high nodule N-fixation rate as the tissue develops. The amount of protein present in the roots was remarkably constant over the whole time course of the experiment being between 2 and 3 mg per g FW. The nodules had higher levels of protein (Fig. 1C) than the parent roots up to 9 weeks, while roots and nodules measured at 11 and 12 weeks had similar protein contents on a fresh weight basis (about 2 mg per g FW).

The presence of different peroxiredoxin isoforms in the nodules during development was investigated using specific antibodies. The plastid 2-Cys Prx and PrxQ isoforms were not detected at any stage of nodule development (data not shown). However, a specific antibody against the cytosolic recombinant 17 kDa *Arabidopsis* Prx II C protein, detected a band of approximately 68 kDa that was most abundant in young nodules and decreased as the nodules senesced (Fig. 5C). As the Prx II C antibody recognises most Prx II isoforms, the detected band is likely to represent a Prx II A homologous protein, which is believed to be encoded by a pseudogene in *Arabidopsis.* The putative AtPrx II A protein is a hybrid protein, which possesses a highly conserved N-terminal Prx II domain and has a total size of 63 kDa [28]. By application of this antibody, Corpas et al. [43] also detected a single 60–70 kDa immunoreactive band in pea peroxisomes. Hence, while the small cytosolic isoforms Prx II B, C and D were not detectable in pea nodules, a putative Prx II A homologous protein is present. A specific antibody against the mitochondrial *Arabidopsis* Prx II F [30] detected a band of approximately 19 kDa. The abundance of this protein remained stable during nodule development and senescence (Fig. 5D).

To determine whether ascorbate levels play an important role in the orchestration of the nodule senescence process, young and old nodules were infiltrated with 20 mM ascorbate. The abundance of the 68 kDa protein was decreased in young

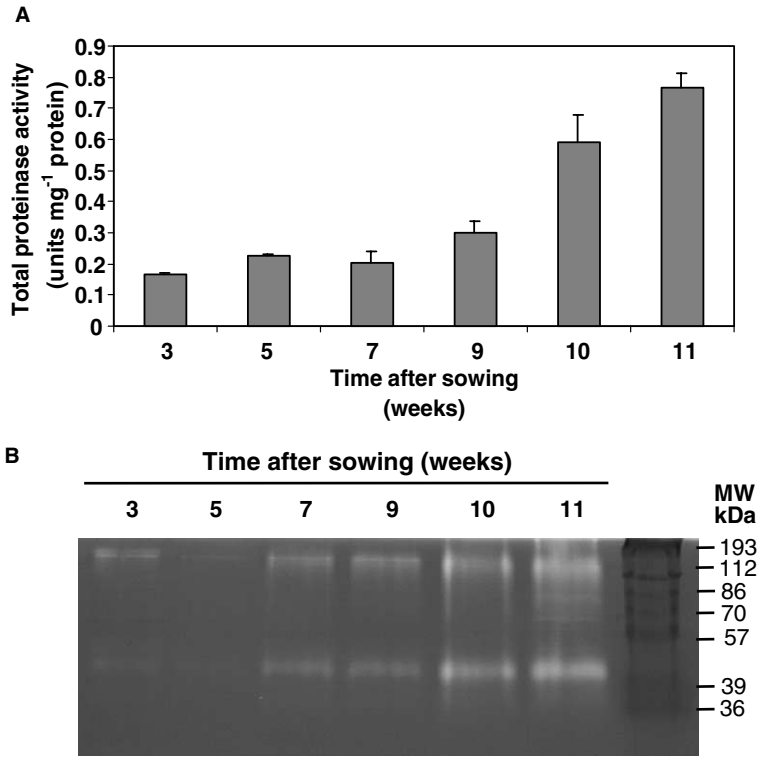

Fig. 2. Proteinase activities during nodule development determined either via the azocaseino-proteinase activity assay (A) or by an in-gel proteinase assay using gelatine as substrate (B).

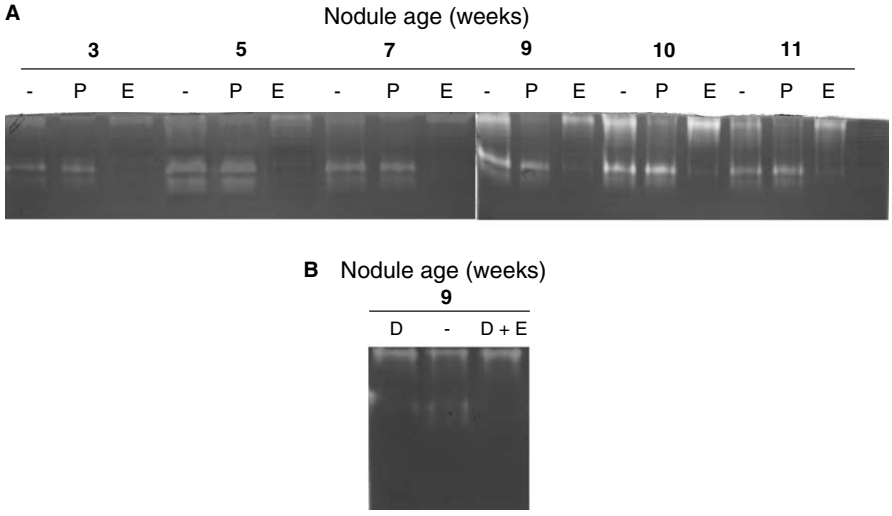

Fig. 3. The effect of class-specific inhibitors and DTT on proteinase activity during nodule development. (A) Extracts were pre-incubated with buffer and assayed at pH 8.0 (–), PMSF (P) or E-64 (E); (B) extracts were pre-incubated with DTT in the presence (D + E) or absence (D) of E-64 and assayed at pH 5.0.

nodules after ascorbate feeding but it was increased in old nodules supplied with ascorbate (Fig. 6A and C). The abundance of the 68 kDa peroxiredoxin was therefore regulated by ascorbate feeding in both young and old nodules. In contrast, the abundance of the mitochondrial Prx II F remained unaltered by these treatments (Fig. 6B and D).

4. Discussion

Nodule senescence is determined by metabolic cues and environmental triggers. Loss of nodule N-fixation capacity began early in development (from 3 weeks after sowing) and occurred together with decreases in nodule ascorbate and glutathione

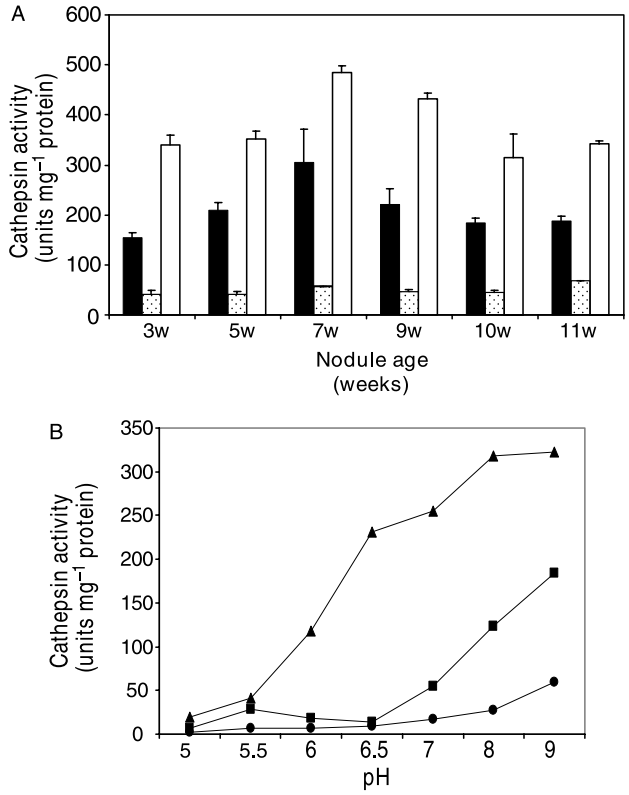

Fig. 4. Changes in cathepsin-like activities during nodule development. A, cathepsin B- (white bar), H- (dotted bar), and L (black bar)-like activities. Each column represents the mean of three plants ± SE; B, cathepsin B- (square), H- (circle), and L (triangle)-like activities with increasing pH of the assay medium.

[27] and well in advance of detectable decrease in leghemoglobin transcripts (data not shown) or changes in leaf chlorophyll or protein (Fig. 1A and B). Decreases in nodule protein occurred at the same time as decreases in leaf protein (Fig. 1B and C), suggesting co-ordination of senescence events occurring in roots and shoots.

Two major protein bands (approximately 45 and 160 kDa) account for the quantitative increase in nodule proteinase activities during senescence (Fig. 2B). While we were unable to obtain these proteins in a pure form for sequencing, we show that the activity of the high molecular weight pea proteinase was completely inhibited by PMSF indicating that it is a serine proteinase, while the second band completely disappeared in the presence of reductant (dithiothreitol) and E-64 (Fig. 3B). Thus, redox-sensitive cysteine proteinase activities, but not cathepsin-like proteinase activities, are up-regulated as part of the nodule senescence programme. These results agree with observations in *M. truncatula* [46], alfalfa [47] and soybean [48].

Ascorbate fulfils signalling functions in plants [44,45]. It is synthesised in the shoot and is imported into the nodule [27]. Nodule azocaseino-proteinolytic activities were low in young nodules and only increased once the nodule ascorbate content had fallen below 300 nmol g^{-1} FW (Fig. 7). The senescence-dependent decline in nodule ascorbate thus appears to favour increased activities of redox-modulated proteinases. Endogenous pools of ascorbate and glutathione decline as pea nodules age [27,49,50]. Moreover, pea nodules cannot undertake de novo ascorbate synthesis [27]. Ascorbate synthesised in the leaves and roots has to be imported into the nodule through the vascular system [27] and decreasing synthesis and export from source organs probably account for the senescence-dependent decrease

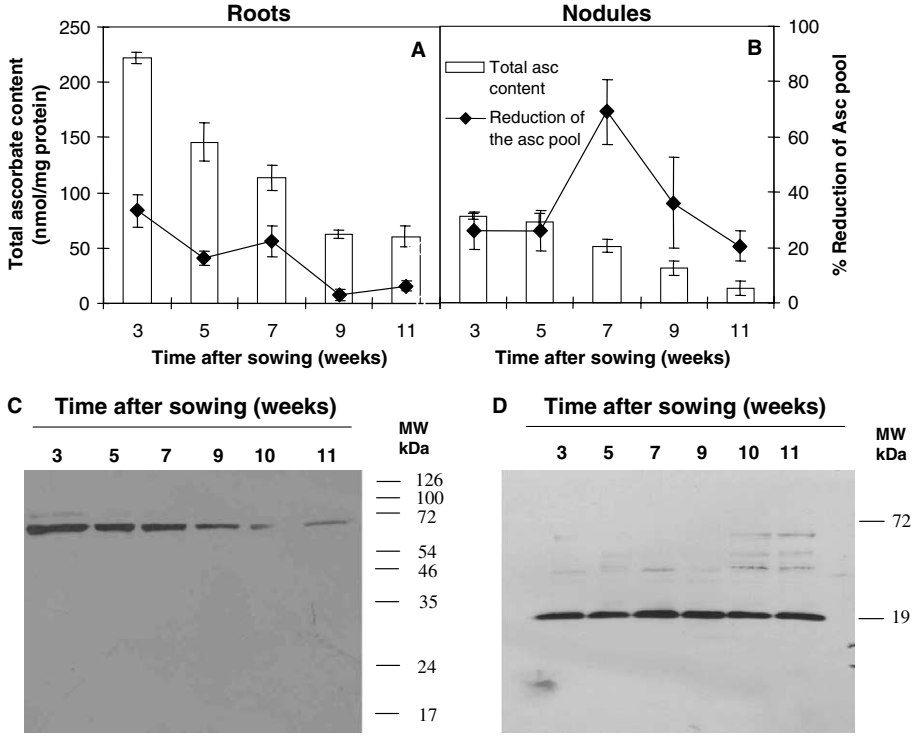

Fig. 5. Developmental changes in the levels of total ascorbate, the ratio of ascorbate to dehydroascorbate and peroxiredoxin protein in pea roots and nodules. Total ascorbate content was measured at the indicated time-points in roots (A) and nodules (B). Each column represents the mean of three plants ± SE. Cytosolic (C) and mitochondrial (D) peroxiredoxin protein content in nodules were detected by immunoblotting with specific antibodies.

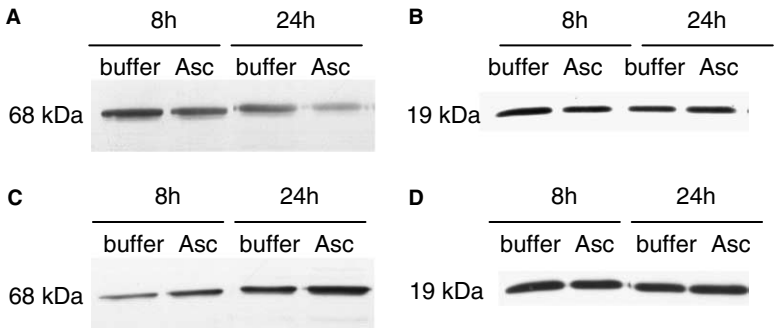

Fig. 6. The effect of ascorbate on peroxiredoxin protein content in nodules. Proteins were extracted from 3-week-old (A, B) or 9-week-old (C, D) nodules supplied with either buffer alone or buffer plus 20 mM ascorbate (Asc) after 8 and 24 h of incubation. The prxII C (A, C) and prxII F (B, D) peroxiredoxin isoforms were detected using specific antibodies.

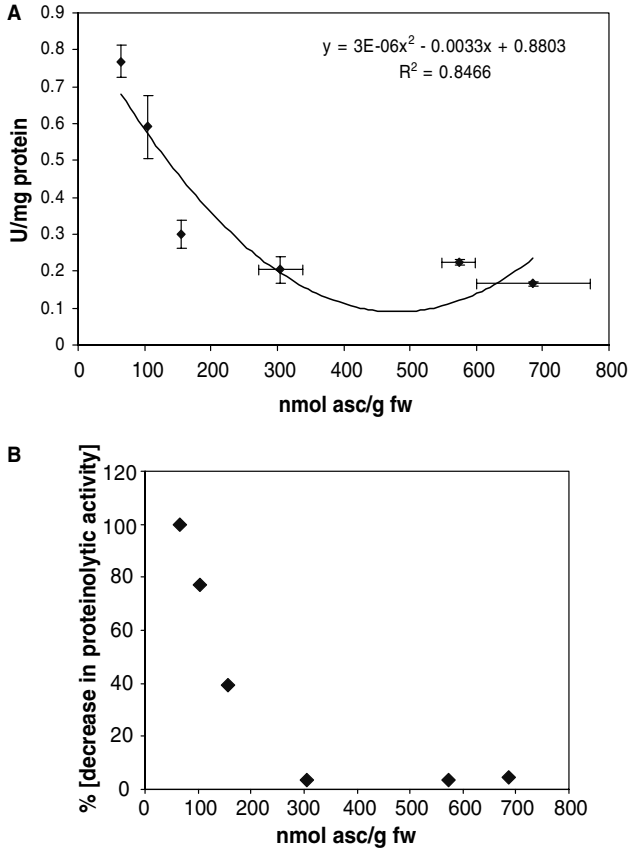

Fig. 7. The relationship between azocaseino-proteinolytic activity and nodule ascorbate content. Nodule tissues were harvested at different stages of development and total ascorbate content and azocaseino-proteinolytic activities were analysed. Each point represents the mean of at least three plants ± SE. Data are plotted either directly (A). or a percentage change in proteinolytic activity (where the highest value observed in senescent nodules was set as 100%; B).

in nodule ascorbate. It is interesting to note that while the percentage of reduced ascorbate decreased in the parent roots as the plants aged, the percentage of reduced ascorbate did not decrease in mature and senescent nodules. However, the proportion of dehydroascorbate measured in roots and nodules was always very high throughout development, suggesting that the ascorbate re-cycling systems are relatively inefficient in roots and nodules.

The results presented here provide the first report of nodule peroxiredoxin proteins. They also provide the first indication that the level of nodule ascorbate can influence the amounts of endogenous peroxiredoxin proteins (Figs. 5 and 6). Peroxiredoxins are ubiquitous non-heme peroxidases differentially transcriptionally regulated by cellular redox status [31]. Transcripts encoding cytosolic Prx II C are specifically up-regulated in *A. thaliana* by feeding ascorbate (Table 1). These cytosolic peroxiredoxins affect plant development and stress tolerance through modified redox signalling and homeostasis (S. Jakob, I. Finkemeier, K.J. Dietz, unpublished results). The putative Prx II 68 kDa nodule isoform identified here was strongly decreased concomitantly with ascorbate during senescence (Fig. 5C). Moreover, the abundance of this isoform was regulated by externally fed ascorbate (Fig. 6). Accumulation of the putative Prx II C protein was induced by external ascorbate in 9-week-old nodules, under conditions where the levels of this Prx II protein and of cellular ascorbate were less than half of those in 3-week-old nodules (Fig. 5C). The response was absent from 3-week-old nodules, suggesting a developmental shift in the control of this peroxiredoxin (Fig. 5A). However, pea nodule mitochondrial Prx II F transcripts were unaffected by either the nodule senescence or by external ascorbate feeding (Fig. 5B and D). These data indicate that mitochondrial peroxiredoxin function is maintained throughout nodule senescence and that it is controlled by factors other than ascorbate in pea nodules.

Cellular redox regulation is central to survival, playing crucial roles in honing and toning diverse signalling pathways used by the plant cell to make appropriate responses to developmental cues and environmental change [51]. The results presented here suggest that the endogenous pools of ascorbate and thiols modulate key nodule proteins. This was observed

Table 1
Effects of oxidants and ascorbate on type II Prx transcript abundance in *Arabidopsis thaliana*

	H_2O_2	Diamide	Ascorbate
PrxII B	0	0	0
PrxII C	+	+	+
PrxII E	0	0	--
PrxII F	0	0	–

Prx II B and C are cytosolic, PrxII E is plastidic and PrxII F is mitochrondrial Increase (+); No change (0); Decrease (–); Strong suppression (--). Data are summarised from [32].

at both the level of protein abundance and turnover (for peroxiredoxin) and also at the level of enzyme activity (azocaseino-proteinolytic activities relative to ascorbate and specifically cysteine proteinases in relation to DTT). The reduction state of protein thiols is a universally important facet of the control of enzyme activity and function in cells. It is important in the ubiquitin-dependent protein degradation pathway [19] that is central to protein degradation events occurring during nodule senescence [52]. Taken together these data illustrate that the senescence-regulated decline in cellular antioxidants such as ascorbate may have a regulatory role in nodule development, decreasing the cellular redox status to favour protein degradation pathways.

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